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Report

Dmp53 Activates the Hippo Pathway to Promote Cell Death in Response to DNA Damage

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Summary

Developmental and environmental signals control a precise program of growth, proliferation, and cell death. This program ensures that animals reach, but do not exceed, their typical size [1]. Understanding how cells sense the limits of tissue size and respond accordingly by exiting the cell cycle or undergoing apoptosis has important implications for both developmental and cancer biology. The Hippo (Hpo) pathway comprises the kinases Hpo and Warts/Lats (Wts), the adaptors Salvador (Sav) and Mob1 as a tumor suppressor (Mats), the cytoskeletal proteins Expanded and Merlin, and the transcriptional cofactor Yorkie (Yki) [2–11]. This pathway has been shown to restrict cell division and promote apoptosis. The caspase repressor DIAP1 appears to be a primary target of the Hpo pathway in cell-death control. Firstly, Hpo promotes DIAP1 phosphorylation, likely decreasing its stability. Secondly, Wts phosphorylates and inactivates Yki, decreasing DIAP1 transcription. Although we understand some of the events downstream of the Hpo kinase, its mode of activation remains mysterious. Here, we show that Hpo can be activated by Ionizing Radiations (IR) in a Dmp53 (*Drosophila melanogaster* p53)-dependent manner and that Hpo is required (though not absolutely) for the cell death response elicited by IR or Dmp53 ectopic expression.

Results and discussion

Hpo is the ortholog of the Mammalian Sterile Twenty-like (MST) kinases, which belong to the Ste20 family of kinases. MSTs are highly similar to Hippo (Hpo) in their N-terminal serine/threonine kinase domains as well as in the C-terminal Salvador (Sav) binding region (or SARAH domain) [12–15]. MST1 functions both downstream and upstream of caspases to promote chromatin condensation and nuclear fragmentation, as well as activation of the JNK (Jun N-terminal kinase) and p38 pathways [16–19]. Like most Ste20 family kinases, MST1/2 auto- or trans-phosphorylates at a number of residues. One of these, T183 in the activation loop, has been shown to be required for full kinase activity and has been used as a useful marker of MST1 activation in cultured cells [20–22]. In order to study events upstream of Hpo, we tested antibodies that have previously been shown to recognize MST1/2 phosphorylated on T183 for their ability to cross-react with Hpo on the equivalent residue (T195). Interestingly, we found antibodies that specifically recognized the phosphorylated form of Hpo upon treatment with staurosporine (sts), a known activator of MST1/2 (Figures 1A; also Figure S1A in the Supplemental Data available with this article online). This signal was abolished by RNAi-mediated Hpo depletion and disappeared upon phosphatase treatment (Figure 1A, fourth lane, and Figure 1C). Moreover, the antibodies recognize overexpressed tagged Hpo before (Figure S1B) or after (Figure S1C) immunoprecipitation. By contrast, the antibodies did not recognize a nonphosphorylable (T195A) Hpo mutant protein (Figure 1B and data not shown). We immunoprecipitated Myc-tagged wild-type and T195A Hpo and measured their auto-kinase activity and their activity on an exogenous substrate (Histone H2B, not shown) in both the presence and absence of sts (Figure 1B). As has been observed for MST1/2, overexpression of Hpo leads to its activation, presumably via trans-phosphorylation. Sts treatment potently stimulated Hpo kinase activity (5-fold). By contrast, the T195A mutant was severely compromised both in its unstimulated and stimulated activities (15- and 10-fold reductions, respectively), suggesting that T195 phosphorylation is crucial to normal Hpo kinase activity, as has been shown for the MSTs [20]. Thus, these phospho-specific antibodies can be used as readouts of Hpo pathway activity.

In the course of testing stimuli that would activate Hpo in tissue culture, we observed that γ-irradiation potently and rapidly induced Hpo activation (Figure 4A). Dmp53, the fly p53 ortholog, has been shown to mediate cell death upon ionizing radiation (IR)-induced DNA damage [23]. Although the pro-apoptotic genes *reaper (rpr)*, *hid*, and *sickle* are Dmp53 transcriptional targets [24, 25], removal of these three proteins via chromosomal deficiencies only partially suppresses the cell-death effects of IR in embryos, suggesting that additional death signals act downstream of Dmp53 [26]. This prompted us to examine whether the Hpo pathway could function downstream of Dmp53 in the response to IR.

First, we treated wing imaginal discs (the larval precursors of the adult wing) containing clones of *hpo, wts*, and *sav* mutant cells with γ-rays and examined cell death by staining for activated caspases (Figure 2). Interestingly, we noted that, although caspase activation was efficiently induced in wild-type tissue (Figures 2D–2F, GFP-positive areas) or control discs (Figure 2B), cell death was severely reduced in *hpo*, *wts*, and *sav* mutant clones (Figure 2, GFP-negative areas) and in *Dmp53* mutant discs (Figure 2C). Quantification of the caspase staining indicated that apoptosis was reduced by 2- to 3-fold in *hpo*, *wts*, and *sav* clones compared to wild-type tissue (Figure 2G; also Figure S4). This was also true in eye imaginal discs (not shown).

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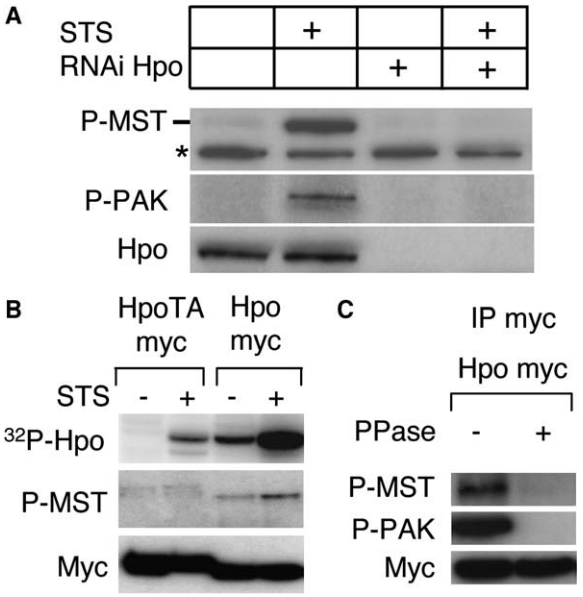

Figure 1. Phospho-Specific Antibodies as Readouts for Hpo Pathway Activity

(A) Antibodies directed against phospho-Mst1 and phospho-PAK1/2 specifically recognize phospho-Hpo upon activating treatment with sts (compare lanes 1 and 2). This is abolished by RNAi-mediated Hpo depletion (lanes 3 and 4). Identical results were obtained with three different *hpo* dsRNAs (not shown). An asterisk denotes a nonspecific band as a loading control.

(B) Wild-type and mutant (T195A) Myc-tagged Hpo were precipitated from Kc cell lysates treated as shown. IP kinase assays were performed in the presence of $^{32}P\gamma$-ATP on nine-tenths of the IPs (top lane), whereas one-tenth were used to check levels of P-Hpo and Hpo-Myc (middle and bottom lanes).

(C) Phosphatase treatment abolishes the phospho-Hpo signal. Transfection of tagged Hpo protein (Hpo-Myc) induces Hpo phosphorylation by trans-phosphorylation. The phospho-Hpo signals disappear upon phosphatase treatment.

Overexpression of Dmp53 in the posterior portion of late larval eye imaginal dics was sufficient to induce apoptosis (Figures 3A and 3B). Loss of function of *hpo, wts*, and *sav* decreased cell death in this context, although the effect was less pronounced in *sav* clones, perhaps as a reflection of the weaker phenotype of the *sav* mutants (Figures 3C–3E). This suggests that the Hpo complex may function as an effector in the Dmp53-mediated response to IR. To test this hypothesis, we first measured Hpo activation in cultured cells treated with γ-rays in the presence or absence of dsRNAs directed against Dmp53 (Figure 4A). Excitingly, depletion of Dmp53 markedly reduced Hpo phosphorylation by IR. The residual level of Hpo activation we observed in Dmp53-depleted cells can probably be explained by the fact that the dsRNA-mediated *Dmp53* depletion was never complete, as measured by RT-PCR (Figure S2A). To check that the increased Hpo phosphorylation we observed corresponded to increased activity, we performed IP kinase assays on cells expressing ectopic Hpo (Figure 4B). We observed that IR treatment potently induced Hpo kinase activity (Figure 4B, compare lanes 1 and 2). Furthermore, *Dmp53* expression alone, in the absence of IR, was sufficient to activate Hpo phosphorylation (Figure 4D, lane 4). Finally, we determined whether Dmp53-dependent Hpo activation could be observed in vivo by taking advantage of the fact that Dmp53 is not required for viability. We treated dissected ovaries from Dmp53 mutant and wild-type flies with γ-rays and examined Hpo activity by Western blotting (Figure 4C). Interestingly, although γ-rays potently activated Hpo in wild-type flies, this response was abolished in *Dmp53* mutant animals. *Dmp53* expression in the ovaries was able to induce apoptosis, ovary degeneration, and total loss of fecundity (Figure S2, compare panels G and H). We conclude that Hpo is activated as part of a Dmp53-dependent DNA-damage response both in cultured cells and in vivo (Figure 4).

MST1 and 2 are known to be activated by caspase 3 through proteolytic cleavage [16, 17]. Therefore, the possibility existed that the Hpo activation we observed was merely a by-product of Rpr-dependent caspase activation. Several lines of evidence suggest that this is not the case. First, *reaper* overexpression in S2 cells did not increase Hpo activity (Figure 4D, sixth lane). Second, depletion of DIAP1 from cultured cells, which potently induces caspase activation, fails to trigger detectable Hpo activation (Figure 4E; also Figures S2B–S2E). Third, the phospho-Hpo signal we detected corresponds to full-length Hpo rather than a caspase-cleaved fragment. In fact, the caspase cleavage site present in the MSTs is not thought to be conserved in Hpo, and we have seen no evidence of Hpo cleavage upon apoptotic stimuli. Fourth, treatment of cultured cells with caspase inhibitors did not affect Hpo activation by IR (data not shown). Thus, it is unlikely that Hpo is stimulated via Dmp53-dependent caspase activation.

The time course of Hpo activation by IR (2–3 hr for maximal activation) suggests that transcription may be required for this response. Indeed, treatment of cells with IR in the presence of the transcription inhibitor Actinomycin D (ActD) abolished Hpo activation (Figure 4F, compare lane 2 with lane 4). Thus, Hpo activation in response to IR requires new gene transcription, which could be mediated, at least in part, by Dmp53. Hpo activity is induced by Dmp53 expression (Figure 4D), but Hpo protein itself does not appear to be a target of Dmp53 because Hpo levels are not detectably upregulated when Dmp53 is expressed in the posterior portion of the eye imaginal disc (Figure S3C) or in Dmp53-expressing clones in the wing disc (Figure S3E). Future studies will be aimed at determining the exact mechanism through which Dmp53 promotes Hpo activation.

In this study, we demonstrate by genetic and biochemical approaches not only that the Hpo pathway is required for the full apoptotic response induced by γ-ray irradiation but also that DNA damage triggers Hpo kinase activity in a Dmp53-dependent manner both in vivo and in vitro. We also show that the apoptosis induced by Dmp53 overexpression is strongly affected in *hpo*, *wts*, and *sav* mutant clones and that Dmp53 does not modulate Hpo levels. This study constitutes the first description of an upstream activating signal of the Hpo complex in vivo and during organism development.

We note that the blockage of Dmp53-induced apoptosis is not complete in *hpo* clones; this incomplete blockage likely reflects the role of other pro-apoptotic proteins, such as Reaper, Hid, and Sickle, in this process.

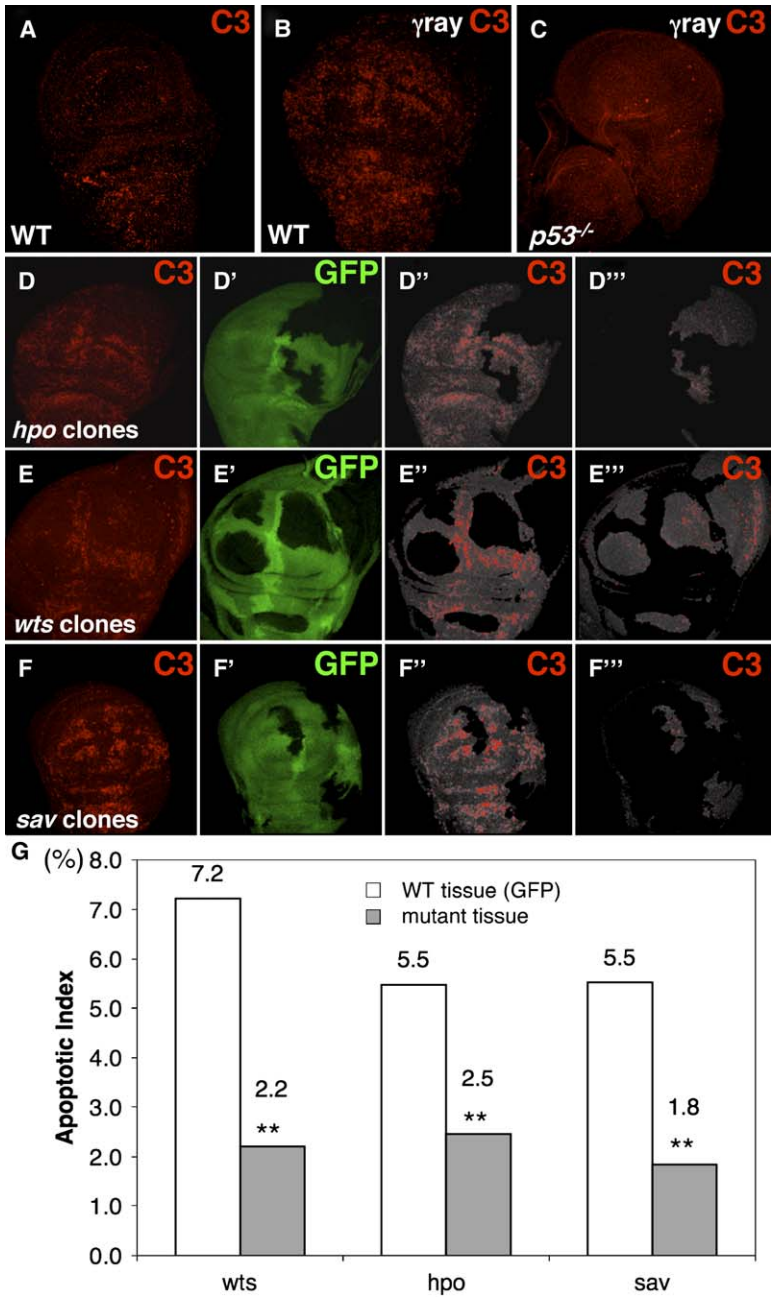

Figure 2. *hpo*, *wts*, and *sav* Mutant Clones Show Reduced IR-Induced Apoptosis

(A–C) 40 Gy of γ rays induces apoptosis after 4 hr, shown by anti-active caspase 3 staining in wing imaginal disks (B) compared to control untreated discs (A). Homozygous $p53^{5A-1-4}$ mutant discs have little detectable γ-ray-induced apoptosis (C). See Experimental Procedures for genotypes.

(D–F) *hpo, wts*, and *sav* mutant clones have reduced γ-ray-induced apoptosis in wing imaginal disks compared to control tissue. hpo^{42-47}, wts^{X1}, and sav^{3} mutant clones are labeled by the absence of GFP (green). Apoptosis is visualized in red by anti-cleaved caspase 3 staining. The GFP signal (D′–F′) was used to separate control (D″–F″) and mutant (D‴–F‴) anti-caspase 3 signals. This approach was used to determine the apoptotic index (see Experimental Procedures) for the various mutants (G). Mutant tissues show a better resistance to γ-ray induced apoptosis. Statistical significance of the difference between wild-type and mutant tissue was assessed by a Mann-Whitney non-parametric test; in all cases, $p < 0.01$ (**) for the null hypothesis. Number of large clones scored for individual genotypes: *wts* n = 28, *hpo* and *sav* n = 34. See Figure S4 and Experimental Procedures for genotypes.

Thus, we propose that, after exposure to ionizing radiations, the dATM, dChk2, Dmp53 signaling pathway [23] is activated and induces apoptosis by targeting expression of pro-apoptotic effectors such as Reaper, as well as by activating the Hpo pathway [24, 25]. This cell-death response to irradiation requires the caspase DRONC and leads to upregulation of JNK activity in a p53-dependent manner [27–30]. Because Hpo has been shown to induce JNK activation when overexpressed in vivo [8], it will be interesting to determine whether Hpo is necessary for IR-induced JNK activation.

Several reports have suggested that the mammalian homologs of members of the Hpo pathway might behave as tumor suppressors in humans [2, 7–9]. In addition, mice lacking the Wts homolog mLats1 are more sensitive to tumor-inducing agents [31]. Our data suggest that one effect of mutations in Hpo-pathway members may be to protect these cells from DNA-damage-induced apoptosis and thus promote tumor progression and the accumulation of additional mutations. Further work on the Hpo pathway should further our understanding of the DNA-damage response and its role in the transformation process.

Experimental Procedures

For *Drosophila* genotypes and further experimental details, see the Supplementary Experimental Procedures.

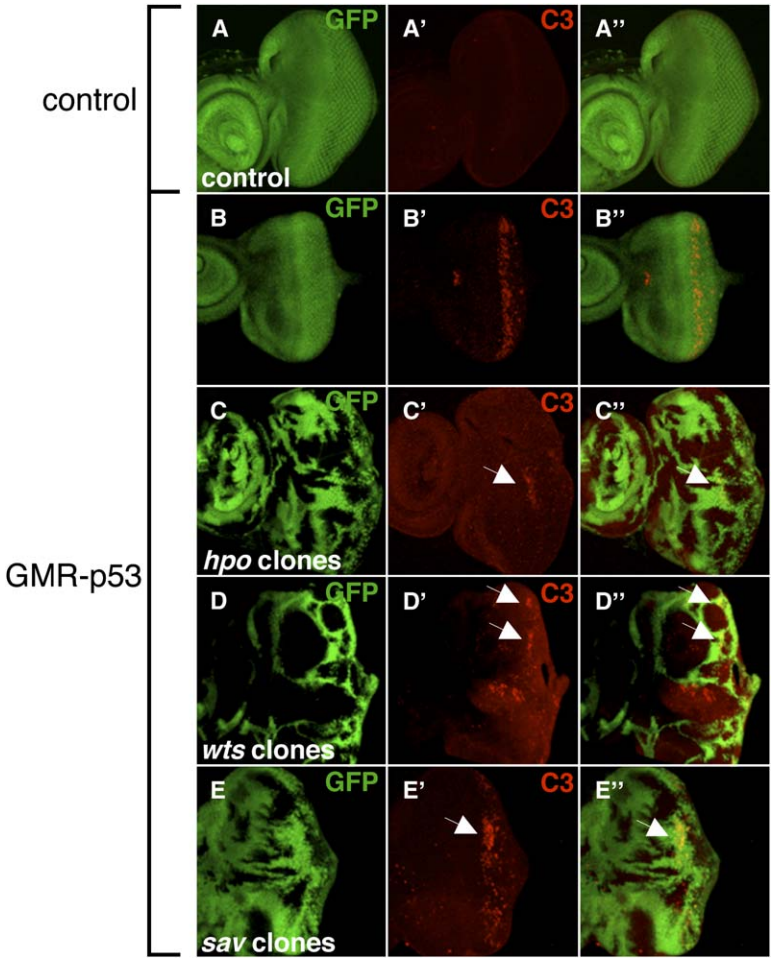

Figure 3. Apoptosis Induced by Dmp53 Overexpression Is Reduced in *hpo*, *wts*, and *sav* Mutant Clones

In all panels, mutant clones are marked by the absence of GFP (A–E and A′–E′). Apoptosis is observed by cleaved caspase 3 staining in red (A′–E′ and A′–D′). GMR (Glass multimer reporter)-driven Dmp53 overexpression in the posterior portion of the imaginal eye discs induces apoptosis (B–B′); compare with the control (A–A′). This is reduced in hpo^{42-47} (C–C′), wts^{X1} (D–D′), and sav^{3} (E–E′) clones. Arrows show that apoptotic cells are mainly wild-type cells (GFP positive). Posterior is to the right. See Experimental Procedures for genotypes.

Kc and S2 Cell Assays

In depletion experiments, *Drosophila* embryonic Kc cells were treated with Dmp53, DIAP1, Hpo, or eGFP dsRNAs for four days. dsRNAs were synthetised with T7 Ribomax large-scale RNA production systems (Promega) and purified with the MEGAclear system (Ambion). We treated Kc cells with 1 μM staurosporine (Sigma) for 3 hr to induce Hpo phosphorylation. To inhibit transcription, we treated Kc cells with 1 μg/ml Actinomycin D (Sigma) for 3 hr.

Western Blotting, Immunoprecipitation, and Hpo Antibody Generation

For Western blotting, we irradiated tissues or cells with γ-rays to induce DNA damage. Tissues were collected 4 hr later, then lysed. After material was cleared, samples containing 50 μg of protein were resolved by SDS-PAGE. Proteins were transferred to PVDF membranes and visualized by immunoblotting. Chemoluminescence was observed via an ECL-plus-Western-blotting detection system (Amersham Biosciences). For all Western-blot experiments, the same membrane was probed first with anti-phospho-Hpo, followed by Hpo, anti-tags, and anti-tubulin antibodies after stripping.

Phosphatase treatments have been done with Lambda Protein Phosphatase (New England Biolabs). Proteins (125 μg) were treated with 500 units of enzyme for 30 min in a 50 μl reaction at 30°C. Immunoprecipitations were done on 250 μg of protein in lysis buffer for 2 hr at 4°C. IP Kinase assays were performed as previously described [7]. Where indicated, 1 μg of *Xenopus laevis* Histone H2B was added to the reaction as a substrate.

The anti-Hpo rabbit antibody was generated and affinity purified by Eurogentec SA against a peptide corresponding to amino acids 191–203.

Genetics and Immunocytochemistry

Mosaic tissues were obtained via the FLP/FRT system. L3 larvae were irradiated with 40 Gy of γ-ray. Four hours after this treatment, tissues were dissected in 1× PBS, fixed in 4% formaldehyde in PBS for 20 min at room temperature, and extensively washed in PBS containing 0.1% Triton X-100 (PBT). Tissues were then blocked for 2 hr in PBT containing 10% goat serum. Primary antibodies were incubated overnight at 4°C. Secondary antibodies were incubated for 2 hr at room temperature at 1/500. After washes, tissues were mounted in Vectashield. Fluorescence images were acquired with a Zeiss LSM510 Meta confocal microscope.

Supplemental Data

Supplemental Experimental Procedures and figures are available with this article online at http://www.current-biology.com/cgi/content/full/16/14/1453/DC1/.

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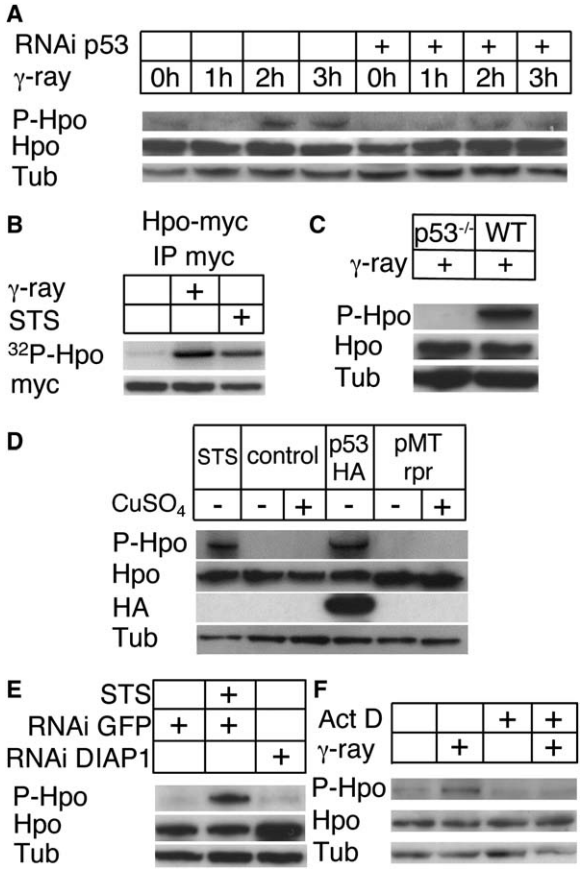

Figure 4. Hpo Activity Is Induced by γ Ray Irradiation both In Vivo and In Vitro in a *Dmp53*-Dependent Manner

(A) Western blot of Kc cell lysates after γ ray treatment with or without Dmp53 depletion by RNAi. Hpo phosphorylation (top row) is induced after 2 hr in RNAi-untreated cells. Dmp53 depletion reduces Hpo activation. Hpo levels (middle row) remain constant in comparison to control levels (anti-tubulin, bottom row). Dmp53 depletion by RNAi treatment is not complete, as measured by RT-PCR (Figure S2A). Times after irradiation are shown in hours.

(B) Myc-tagged Hpo was immunoprecipitated from Kc cells treated as shown. IP kinase assays were performed in the presence of ^{32}P γ-ATP on nine-tenths of the IPs (top lane), whereas one-tenth were used to check levels of Hpo-Myc (bottom lane). Hpo kinase activity is strongly induced by IR treatment (compare lanes 1 and 2).

(C) Western blot of γ-ray (40 Gy)-treated Dmp53 mutant or wild-type ovaries 4 hr after irradiation. Hpo phosphorylation is severely reduced in Dmp53 mutant animals (top row). Hpo levels (middle row) and tubulin levels (bottom row) are shown.

(D) Western blot of S2 cell lysates after transfection of Dmp53 or induction of *reaper* expression. Hpo phosphorylation (top row) is induced by *Dmp53* expression (fourth lane) but not by *rpr* expression (sixth lane). Induction by $CuSO_4$ has no effect on Hpo activity (third lane). Hpo levels (second row), HA levels (third row), and tubulin levels (bottom row) are shown. P-Hpo was also induced by IR alone in S2 cells (not shown).

(E) Western blot of Kc cells treated with dsRNAs to deplete DIAP1. Hpo phosphorylation is induced by sts treatment (compare top row in lanes 1 and 2). DIAP1 depletion fails to induce Hpo phosphorylation (top row, lanes 3). As expected, DIAP1 depletion potently induces cell death (see Figures S2C–S2E). Hpo levels (middle row) and tubulin levels (bottom row) are shown.

(F) Western blots of γ-ray-treated or control Kc cells in the presence or absence of 1 μg/ml Actinomycin D. γ-ray-induced Hpo phosphorylation is severely reduced in the presence of Act D (compare lanes 2 and 4). Hpo levels (middle row) and tubulin levels (bottom row) are shown.

UK. We apologize to colleagues whose work we could not cite due to space constraints.

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